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MALDI-TOF MSI method for determining spatial distribution of infection markers in pulmonary tissues of pigs

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Abstract: In recent years the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI-TOF MSI) is used for molecular mapping of diverse biomarkers such as proteins or peptides in animal/plant tissue sections. It takes full advantage of the benefits of MALDI-TOF technique which is the ability of rapid measurements of all mass spectra in a wide mass range and detection of analytes molecular weights. Interleukins, the group of mostly proinflammatory cytokines, are the proteins that are produced as immune response on bacterial infection *Actinobacillus pleuropneumonia*. The aim of this study was to develop a MALDI-TOF MSI method for quantitative visualization of spatial distribution of interleukins and other cell markers of lymphocytes, granulocytes and macrophages in porcine tissues of lymph nodes and lungs. The determination of the spatial distribution of produced proteins will bring further useful knowledge of the pathogenesis of this economically important disease of pigs, which can also contribute to reducing the consumption of antimicrobials.

Key Words: mass spectrometry imaging, cytokines, interleukins, infection, pig model

INTRODUCTION

Actinobacillus pleuropneumoniae (APP) belongs among the most important bacterial pulmonary pathogens in pigs of all ages and is found worldwide. APP is gram-negative bacteria from the family *Pasteurellaceae* that causes swine disease called porcine pleuropneumonia that is characterized as an exudative, fibrinous, hemorrhagic, and necrotizing pleuropneumonia (Zimmerman et al. 2012). This bacterial infection mainly affects the lung parenchymal tissue of the animal. The clinical course of disease can vary from per-acute to chronic depending on the serotype of infection, the immune status of the host, and the number of bacteria reaching the lung. Clinical signs during per-acute or acute disease include high fever, increased respiratory rate, coughing/sneezing, dyspnea, anorexia, ataxia, vomiting, diarrhoea, and severe respiratory distress with cyanosis. These symptoms of disease negatively affect the economy of the breeding itself (Bossé et al. 2002).

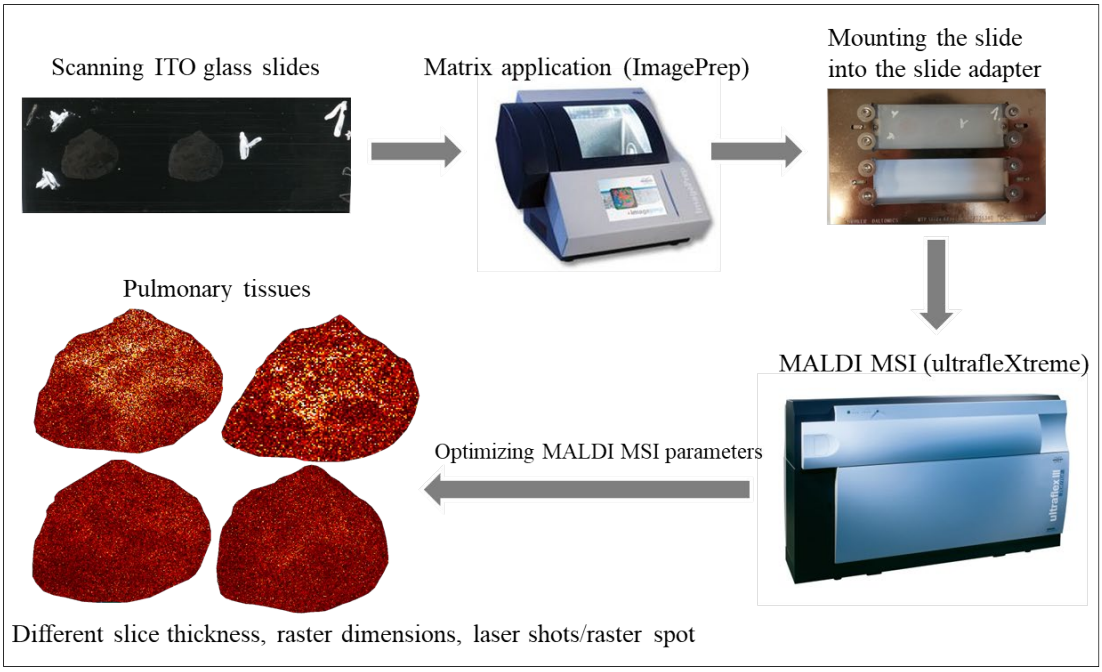
Virulence of APP is multifactorial as in most pathogenic bacteria. Virulence factors of APP include the capsular polysaccharides, lipopolysaccharides, membrane proteins, adhesion factors and exotoxins (Frey 1995). The immune response of organism is local production of the cytokines interleukin (IL)-1β, IL-8, IL-6 and tumor necrosis factor (TNF)-α in porcine lungs (Ondrackova et al. 2010, Hsu et al. 2016).

Holzlechner and co-workers showed that MALDI-MSI can be used as tool for the characterization and *in situ* localization of immune cell accumulations without using antibodies, which is based on

protein expression in these cells. They also detect *m/z* values allowing for identification and discrimination of lymphocytes, monocytes, and polarized macrophages by intact cell mass spectrometry (ICMS) (Holzlechner et al. 2017).

The matrix assisted laser desorption/ionization technique is routinely used for the analysis of peptides, proteins and identification of bacteria. One of the characteristics of MALDI is soft ionization of biomolecules that allows to generate mostly singly charged ions when short UV laser pulses are fired onto the surface with sample and matrix. Due to soft ionization of biomolecules, MALDI was found as useful tool for mass spectrometry imaging of a variety of samples providing us the information about the spatial distribution of molecules. MALDI MSI was first used for molecular mapping of protein expression in human and mice tissues of brain (Stoeckli et al. 2001). The typical procedure for MALDI MSI on tissues consists of collecting thin tissue section using a cryostat (Tucker et al. 2011), attaching section on a sample plate, and depositing of a matrix, either as a thin layer, or as a spot pattern. In this approach, for co-crystallization with analytes is usually used as a matrix a weak organic acid such as sinapinic acid or 2,5-dihydroxybenzoic acid. The sample plate with section of tissue is then introduced in the mass spectrometer and both the intensities of *m/z* values and their respective x/y positions within a sample are acquired. From a data set, numerous images representing the spatial distribution of the analytes of interest can be obtained. Usually, the molecular weight of analytes are determined using a time-of-flight (TOF) mass analyzer (Rohner et al. 2005). Currently, it is effort to develop MALDI MSI technique in different ways as time of analysis (Baker et al. 2017), spatial resolution (Baker et al. 2017, Huang et al. 2018) and the ionization efficiency and detection of different analytes (Hansen and Lee 2018). A schematic general workflow of the MALDI MSI is described in Figure 1.

Figure 1 Scheme of general workflow of MALDI mass spectrometry imaging.



In the present study, we have focused on optimizing the MALDI-TOF mass spectrometry imaging of interleukins and other cell markers of lymphocytes, granulocytes and macrophages in porcine tissues of lymph nodes and lungs. The results from this study will help us in future experiments with cytokines in different tissues.

MATERIAL AND METHODS

Materials

Sinapinic acid (SA) and all solvents (HPLC grade) used were purchased from Sigma-Aldrich (MO, USA) if not otherwise stated. Conductive indium-tin oxide (ITO) one-side coated glass slides and protein calibration standards were purchased from Bruker Daltonik GmbH (Germany).

Collection and Processing of Lymphatic and Pulmonary Tissue

Pieces of lymphatic and pulmonary tissues were taken from infected pigs by APP. Pig breeding was carried out at accredited experimental stables of Veterinary Research Institute in Brno (authorization to use experimental animals, file no. 58809/2014-MZE-17214, valid until 21. 8. 2019). This accreditation also allows to experimentally infect animals under controlled conditions. The experiment was performed in compliance with the Act No. 246/1992 Coll. of the Czech National Council on the protection of animals against cruelty, and with the agreement of the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (approval no. 31674/2018-MZE-17214).

The infection with APP (field-origin strain, biotype 1, serotype 9, KL2-2000) was performed intranasally during inhalation, and the infectious dose of $2x\ 10^9$ bacteria was administered to the second third of each nasal cavity as described previously (Ondrackova et al. 2013). After euthanasia of the animals, samples of tracheobronchial lymph nodes and affected lung tissue were taken for subsequent laboratory analyses.

Cryosections of pulmonary tissue were prepared according to the following protocol *preparing a cryostat section for MALDI imaging* from FlexImaging 3.0 User Manual. The samples were cut to a thickness of 5–10 µm by the cryostat (Leica Microsystems, CM 1900, GmbH, Wetzlar, Germany) at temperature -20 °C. Cryosections for MALDI MSI were mounted onto ITO glass slides and stored at -80 °C.

Prior to analysis, the slides were warmed by hand and desiccated under vacuum for 15 min. Then, the slides were washed in a Coplin jar with ethanol (twice in 70% ethanol for 2 min and once in 100% ethanol for 2 min). The matrix application samples were dried under vacuum for 15 min, and the positions of the tissue slices were marked with three guide marks using a white pencil corrector. Afterwards, the glass slides were scanned by an Epson Perfection V500 Office scanner (Epson Europe B.V., Netherlands) at a resolution of 3200 DPI.

MALDI matrix was sprayed onto ITO glass slides using ImagePrepTM standard programs (Bruker Daltonik GmbH, Germany). Sinapinic acid (Sigma-Aldrich, St. Louis, MO, USA) was used as MALDI matrix. SA was prepared in concentration of 10 mg/ml in 60% acetonitrile and 0.2% trifluoroacetic acid (TFA). MALDI matrix mixtures were thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Berlin, Germany) for 2 minutes at 50% of intensity at room temperature. The samples were ready for analysis after drying.

MALDI MSI analysis

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany) using a protocol according to (Guran et al. 2017). The total sample set consisted of 2 ITO glass slides containing 4 tissue sections. The scanned images of tissue slices were loaded into FlexImaging 3.0 software (Bruker Daltonik GmbH, Germany), and a MALDI adapter with two ITO glass slides was loaded into the mass spectrometer. The position of the MALDI adapter was adjusted according to the white guide marks on the ITO glass slides. The regions of acquisition were highlighted by the mouse pointer in FlexImaging, and 50 µm and 100 µm raster width were chosen. External calibration was performed using a protein standard mixture in an *m/z* range of 4–20 kDa. The intensity of each scan over the entire acquired mass range was mapped on the tissue section image to visualize the location of each *m/z* value detected. These images were generated and visualized using SCiLS Lab 2014b software (SCiLS–Bruker Daltonik GmbH, Germany). The laser power was set to 85% for the SA matrix. MALDI MSI of proteins was performed in linear positive mode in a *m/z* range of 4–20 kDa. A total of 1000 spectra were summed for each spot using a random walk raster pattern, with no evaluation criteria.

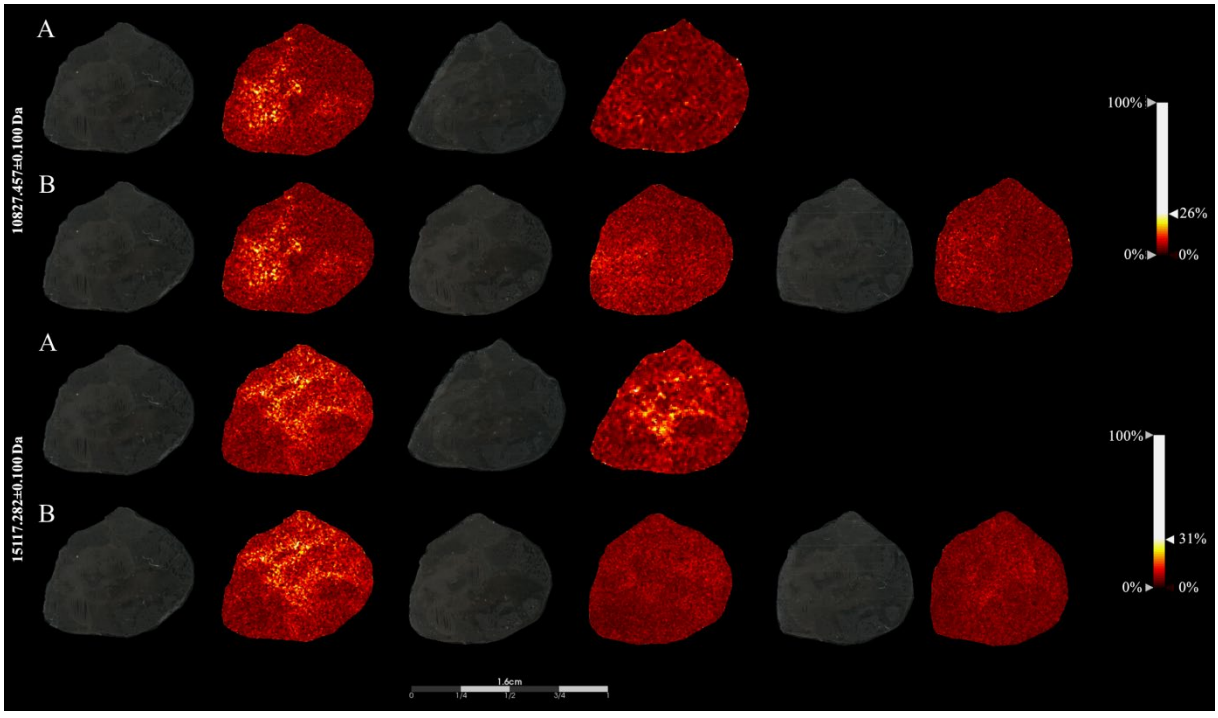
Spectral processing and statistics

The MSI data from FlexImaging were converted and uploaded into the SCiLS Lab software used for pipeline segmentation and statistical analysis (namely the Anderson-Darling normality test and the Kruskal-Wallis test).

RESULTS AND DISCUSSION

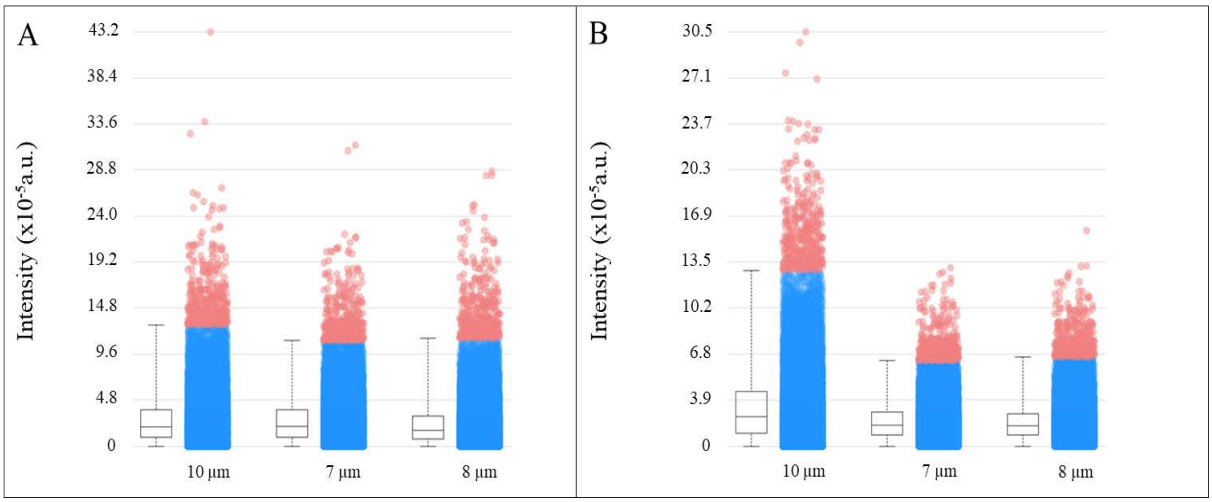
In the study (Holzlechner et al. 2017), they were able to visualize the localization of lymphocytes and macrophages in human colon tissue sections by MALDI MSI. Therefore, we decided to utilize and optimize the MALDI-TOF MSI method to obtain spatial (2D) distribution of interleukins and other cell markers of lymphocytes, granulocytes and macrophages in porcine tissues of lymph nodes and lungs for our future research. In the present work the tissue thickness and laser shots per raster spot were optimized. Sections of various thickness (from 7 to 10 µm), rasters of various dimensions (50×50, 100×100) and different numbers of laser shots per raster spot (300, 500, 1000 and 1200 laser shots per raster spot) were tested. For analysis were used cryo-sectioned frozen tissue samples because there are no other interferences for MALDI-TOF mass spectrometry. Also can be used formalin-fixed and paraffin-embedded (FFPE) sample, but preparation of FFPE sample is more complicated. Paraffin can suppress ionization and formaldehyde fixation causes dehydration, denaturation, crosslinking (methylene bridges), precipitation and protein agglutination, which prevents their detection (Guran et al. 2016). Prior to protein MSI analysis, the samples were processed using a solvent washing step. The MSI data were then imported into SCiLS Lab software for post-processing and generation of protein profiles and specific ion maps. A several peaks with notable signal intensity were generated. For an example, the peaks at *m/z* 10827.457 and 15117.282 Da, that should represent cell markers of lymphocytes or monocytes according study (Holzlechner et al. 2017), were found in each pulmonary tissues. These peaks were selected to generate MSI images (Figure 2) and intensity box plots (Figure 3) to view the differences among various raster spots and various thicknesses of tissue sections. The optimal size of a raster spot was 50×50 and the optimal number of laser shots per raster spots was determined to be at least 500.

*Figure 2 MALDI ion images of selected m/z values (10827.457 and 15117.282 Da) representing markers of monocytes and lymphocytes in pulmonary tissue with various (**A**) size of a raster spot (50×50, 100×100) and various (**B**) thicknesses of cryosections (10, 7, 8 µm).*



The MSI data were submitted to SCiLS Lab where were revealed significant ($p < 0.001$) differences among tissues by the Kruskal-Wallis test. The peak at *m/z* 15117.282 Da varied significantly ($p < 0.001$) among pulmonary tissue with various thickness sections. The peak at *m/z* 10827.457 Da varied significantly ($p < 0.001$) among sections unlike between sections with thickness 10 µm and 7 µm ($p \geq 0.05$, $r = 0.89$). Higher intensities of the peaks at m/z 10827.457 and 15117.282 Da were found in section with thickness 10 µm according the intensity box plots.

*Figure 3 Intensity box plots of 10827.457 Da (**A**) and 15117.282 Da (**B**) of pulmonary tissue with various thicknesses of cryosections.*



CONCLUSION

Interleukins are of special interest as they have been shown to be involved in disease called porcine pleuropneumonia. This study demonstrates that MALDI MSI method was optimized to successfully visualize the spatial distribution of a several peaks, for an example the peaks at m/z 10827.457 and 15117.282 Da, representing interleukins and other cell markers of lymphocytes, granulocytes and macrophages in porcine cryo-sectioned frozen tissues of lymph nodes and lungs. This result is promising for future research with cytokines in different tissues.

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